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14. ABSTRACT Ets proteins regulate expression of genes involved in tumorigenesis. In lung cancer, increased Ets1 expression is associated with poor prognosis. We hypothesized that Ets1 contributes to lung tumorigenesis by binding to specific promoters that control transcription of genes involved in EMT, such as Twist1. We used a mouse lung cancer model with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss (KrasG12D/Lkb1L/L model) and a similar model but one that does not metastasize, driven by conditionally activated Kras alone (KrasG12D model). We measured expression of Twist in KrasG12D /Lkb1L/L and KrasG12D cell lines. Using ChIP assays, we determined whether Ets1 binds Twist1. We determined whether silencing Ets1 decreases Twist1 expression. Ets1 and Twist1 differ in gene expression between KrasG12D (low Ets1 and Twist1) and KrasG12D/Lkb1L/L (high Ets1 and Twist1) tumors. In human lung tumors, Twist1 and Ets1 staining positively correlate. ChIP assays confirm binding of Ets1 to the Twist1 promoter. Silencing Ets1 decreases Twist1 expression and decreases migration and invasion. Using both mouse and human lung cancer cell lines, we show that Ets1 regulates the expression of Twist1. Therapeutic targeting of EMT regulators could be useful to impair tumor metastasis.				
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Identification and Function of Ets Target Genes Involved in Lung Cancer Progression

Introduction: Ets proteins are extremely important in human cancer progression (Seth and Watson, 2005). 27 human Ets family members have been identified, and in some systems multiple Ets factors act in concert to regulate pathways involved in tumorigenesis (Turner et al., 2007). One model of metastasis proposes that a subset of tumor cells within epithelial malignancies acquires the ability to disseminate by undergoing an epithelial-mesenchymal transition (EMT). These cells are characterized by (1) loss of cell-cell attachments, E-cadherin expression, and apical-basal polarity, and (2) appearance of mesenchymal differentiation properties (Huber et al., 2005). We hypothesize that Ets1 contributes to lung tumorigenesis by binding to specific promoters that control transcription of genes involved in EMT. To test our hypothesis, we used a mouse lung cancer model with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss (Kras^{G12D} /Lkb1^{L/L} model) and a similar model but one that does not metastasize, driven by conditionally activated Kras alone (Kras^{G12D} model). We measured expression of Ets1 and Twist in Kras^{G12D} /Lkb1^{L/L} and Kras^{G12D} cell lines. Using ChIP assays, we determined whether Ets1 binds Twist1. We determined whether silencing Ets1 decreases Twist1 expression. Our findings to date are summarized in this report.

Body: To test our hypothesis that Ets1 contributes to lung tumorigenesis by binding to specific promoters that control transcription of genes involved in EMT, we proposed the specific aims below. Our progress and findings associated with each specific aim are outlined below. For detailed description of methods, figures, and figure legends, please see Appendix 3 (submitted manuscript) and Supporting Data.

SA1. Determine the mechanism of increased Ets1 expression in the metastatic Kras G12D /Lkb1L/L model compared to the non-metastatic Kras G12D model.

1a. Determine whether the inactivation of Lkb1 increases the induction and protein

stability of Ets1 and Pea3.

We confirm that murine Kras^{G12D} /Lkb1^{L/L} primary tumors and metastases, compared to Kras^{G12D} primary tumors, have increased Ets1, but not Pea3, mRNA by microarray analysis and both increased Ets1 and Pea3 protein expression by immunohistochemistry. Gene expression microarray analysis shows increased Ets1 expression in Kras^{G12D} /Lkb1^{L/L} primary tumors and metastases compared to Kras^{G12D} primary tumors (Fig 1a). To determine if cell types expressing Ets1 differ in the two models, we performed immunohistochemistry on Kras^{G12D} and Kras^{G12D} /Lkb1^{L/L} specimens (Fig 1b). In Kras^{G12D} primary tumors, Ets1 protein expression could not be detected. In Kras^{G12D}/Lkb1^{L/L} primary tumors and metastatic lymph nodes, scattered epithelial cancer cells express Ets1.

Although Pea3 is expressed in both models, there were no differences in Pea3 mRNA expression between Kras^{G12D} and Kras^{G12D} /Lkb1^{L/L} specimens (Fig 1a). Since differences in mRNA expression were not detected in Pea3 both by microarray gene expression analysis and QRT-PCR, our focus in SA2 and SA3 focused on Ets1.

1b. Characterize regulatory elements in the Ets1 promoter and identify transcription factors, including Pea3, that preferentially transactivate Ets1 in tumor cells derived from the Kras^{G12D} /Lkb1^{L/L} compared to the Kras^{G12D} model.

We determined that since Pea3 expression by gene expression microarray analysis, QRT-PCR and protein expression did not differ between the 2 mouse models, that Pea3 is likely to not directly transactivate Ets1 in lung cancer metastases.

SA2. Determine whether Ets1 and Pea3 are necessary for EMT and metastasis to lymph nodes for lung cancer cells with inactive Lkb1.

2a. Using ChIP assays, determine whether Ets1 and Pea3 directly (or indirectly) bind and regulate predicted EMT targets in cell lines derived from Kras^{G12D} tumors and Kras^{G12D} /Lkb1^{L/L} tumors and LNs.

Using cell lines derived from $Kras^{G12D}/Lkb1^{L/L}$ tumors and lymph nodes, we show that Ets1 binds to the mouse Twist1 promoter. We used GSEA and Transfac databases to determine if gene expression differences between $Kras^{G12D}/Lkb1^{L/L}$ versus $Kras^{G12D}$ models were enriched in putative Ets1 targets. We found that Twist1, an Ets1 predicted target, is expressed at higher levels in $Kras^{G12D}/Lkb1^{L/L}$ compared to $Kras^{G12D}$ primary tumors. In the proximal 2 kb human Twist1 promoter, there are 11 putative Ets sites and in the murine Twist1 promoter there are 34 Ets sites (Qin et al., 2009; Howe et al., 2003).

Cell lines derived from $Kras^{G12D}/Lkb1^{L/L}$ (KW-821) and $Kras^{G12D}$ (KW-634) tumors markedly differ in Twist1 mRNA levels (Fig 2a). KW-821 cells express higher levels of Twist1 mRNA compared to KW-634 cells by QRT-PCR. We performed ChIP assays in KW-821T and KW-821LN cell lines (Fig 2b). Immunoprecipitated samples using IgG or Ets1 antibodies show that Ets1 binds to the Twist1 promoter in both KW-821T and KW-821LN cells. For negative control (Twist1 promoter sequences to which Ets1 does not bind), there is no difference between IgG and Ets1.

2b. Determine whether silencing Ets proteins and their targets, such as Twist, (1) alters expression of genes known to be involved in EMT and (2) modulates in vitro metastatic potential by performing standard migration and invasion assays using $Kras^{G12D}/Lkb1^{L/L}$ and human lung cancer cell lines.

Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels. siRNA mixture targeting Ets1 or non-targeting siRNA control were transfected into KW-821T and KW-821LN cells. We confirm effective knockdown of Ets1 by QRT-PCR and western analysis (Fig 3). Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels in both cell lines (Fig 3).

Silencing either Twist1 or Ets1 decreases migration and invasion in cell lines derived from $Kras^{G12D}/Lkb1^{L/L}$ primary and metastatic cells. Cells were transfected with siRNAs (siRNA-Ets1, siRNA-Twist1, and siRNA-control), and then seeded in Matrigel-coated invasion or migration chambers for 24 h. Extent of invasion (A) and migration (B)

were expressed as an average number of cells per microscopic field. Silencing Twist1 or Ets1 decreases migration and invasion in KW-821 (T and LN) cells (Fig 4).

2c. By crossing Ets1 -/- mice (and/or their targets) with Kras^{G12D}/Lkb1^{L/L} mice, determine in an in vivo model of lung tumorigenesis whether silencing these Ets proteins impede progression to highly invasive and metastatic disease.

Our biggest accomplishment during these past 3 years was that from our preliminary data that we generated from this award, we were able to conceptualize in year 3 that we now have the tools to develop a sensitive method to lineage-tag and track lung epithelial cells in a mouse model of metastatic lung cancer, thereby allowing us to determine the initial steps of the metastatic cell in lung cancer. Instead of the proposed crossing outlined in 2c, we realized that by interbreeding Nkx2.1-CreERT2 knock-in mouse strain (4), containing a tamoxifen-inducible lineage specific Cre recombinase, with Kras^{G12D}/Lkb1^{L/L} mice to generate adult lung-specific mutations in Kras and Lkb1 and introducing a RosaYFP (lox-stop-lox-YFP) into the mutant background, we could specifically label and track lung epithelial cells during tumor progression and metastasis. Successful generation of this mouse model [KrasG12D(L/-)Lkb1(L/L);RosaYFP(L/-);Nkx2-1-creER(+/-)] could shift the current paradigm of lung cancer metastasis and aid in early detection and novel treatment approaches. Since this model would take over a year to generate and is not within the scope or aims of this proposal, we applied for the DOD concept grant to generate this model. We were awarded this grant and as of 9/15/2013 we are beginning our work to generate this model.

SA3. Determine whether Lkb1 mutations associate with Ets1 and Pea3 expression and activity in tumors of lung cancer patients.

3a. Using human lung cancer specimens, determine whether Ets1 and Pea3 are differentially expressed between patients with and without the Lkb1 inactivating mutation using QRT-PCR, western blot, and immunohistochemistry (IHC).

30% of human lung carcinomas have loss of LKB1. Overall 5 year survival is decreased in patients with Lkb1 loss. We show that Twist1 staining positively correlates with Ets1 immunostaining in human primary tumor specimens. We characterized the expression pattern of Ets1 and Twist1 in 10 primary tumors (5 primary tumors without LN metastasis; 5 primary tumors with LN metastasis). In primary tumors the expression pattern of Twist1 and Ets1 positively correlate. In Twist1-positive stromal cells, Ets1 expression is detected in stromal cells. In Twist1-positive tumor epithelial nuclei, Ets1 is detected in tumor epithelial nuclei (Fig 5).

3b. By comparing human lung cancer specimens from patients with and without the Lkb1 inactivating mutation, determine in vivo by ChIP assays and QRT-PCR if there is (1) differential binding of Pea3 and Ets1 to target promoters involved in EMT (such as Twist) and (2) expression of these target genes.

We performed ChIP assays in human (A549 and NCI-H1299) cell lines to confirm Ets protein binding to the human Twist1 promoter (Fig 6). Sonicated cell extracts were immunoprecipitated using IgG or Ets1 antibodies and analyzed by PCR for the Twist1 promoter spanning the Ets1 site. Figure 6 is a representative gel showing that Ets1 binds to the Twist1 promoter in both cell lines. For negative control (Twist1 promoter sequences to which Ets1 do not bind), there is no difference between IgG and specific antibodies. Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels. Taken together, ChIP and siRNA data confirm that Ets1 is a transcriptional activator of Twist1 in lung cancer cell lines.

In patients with adenocarcinomas, Twist expression is correlated with differentiation status of lung adenocarcinomas. Kruskal-Wallis analysis on Twist positive cases showed a significant difference among five histologic subtypes ($P < 0.0001$). The most common subtype was Acinar followed by Solid. Chi-square analyses revealed a significant difference in Twist expression in cases that comprise greater than 20% of either or both of these two subtypes

Key Research Accomplishments

- We show for the first time, that in both human and murine lung cancer cell lines, Ets1 regulates Twist1 expression.
 - We confirm increased Ets1, but not Pea3, mRNA expression by QRT-PCR in Kras^{G12D}/Lkb1^{L/L} (metastatic mouse model) compared to Kras^{G12D} (non-metastatic mouse model) primary tumors.
 - We determined by ChIP assays that Ets1 binds to the Twist1 promoter in mouse and human cell lines.
 - Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein indicating that Ets1 is likely a direct transcriptional activator of Twist1 in lung cancer cell lines.
- We characterized the expression pattern of Ets1 and Twist1 in primary tumors and metastatic lymph nodes. Twist1 staining positively correlates with Ets1 immunostaining in both murine and human tissues. Furthermore, Ets1 and Twist1 protein expression match in terms of expressing cells.
- We show that silencing Twist1 or Ets1 decreases migration and invasion in both KrasG12D /Lkb1L/L primary and metastatic-derived cell lines.
- In human lung specimens, Twist expression is correlated with differentiation status of lung adenocarcinomas.

Reportable Outcomes

American Thoracic Society meeting invited oral presentation (5/2011)

“Ets-1 Regulates Twist-1 Expression In Non-Small Cell Lung Cancer (NSCLC) Progression and Metastasis”

K.K. Wong, MD, PhD, J. Carretero, PhD, M.I. Ramirez PhD, J. Li, A. Hinds, M.C. Williams, PhD, , and H Kathuria, MD. Pulmonary Center, Department of Medicine, BUSM, Boston, MA, United States.

American Thoracic Society, Section on Thoracic Oncology (ATS SOTO) annual Research Excellence Award (5/2011)

Recognition of abstract titled “Ets-1 Regulates Twist-1 Expression In Non-Small Cell Lung Cancer (NSCLC) Progression and Metastasis”

United States and Canadian Academy of Pathology (USCAP) Abstract Presentation (2/2011)

“Correlation of Twist Expression in Primary Non Small Cell Lung Carcinomas with Histologic Subtypes, Risk of Metastasis, and Overall Patient Survival”

Katherine J. Downey, MD; Yanelba Toribio, MD; Hasmeena Kathuria, MD; Benedict Daly, MD; and Carl O'Hara, MD

Manuscript in preparation (10/2013)

“Ets-1 Regulates Twist-1 Expression In Non-Small Cell Lung Cancer (NSCLC) Progression and Metastasis”.

Jun Li¹, Julian Carretero², Carl J O'Hara³, Anne Hinds¹, Guetchyn Millien¹, Mary C Williams¹, Kwok-Kin Wong^{2, 4}, Hasmeena Kathuria MD¹

Department of Defense Concept Award (W81XWH-13-1-0184) (9/15/2013)

Title of Project: In Vivo Tagging of Lung Epithelial Cells to Define the Early Steps of Tumor Cell Dissemination

United States and Canadian Academy of Pathology (USCAP) Abstract Presentation

2014 USCAP Annual Meeting in San Diego, CA, March 1-7

“Correlation of Twist Expression in Different Histologic Subtypes of Primary
Adenocarcinoma in Lung”

Anita Malek , MD; Hasmeena Kathuria, MD; and Carl O'Hara, MD

Conclusions

In this progress report, we show that in mouse and human lung tumors, Twist1 staining positively correlates with Ets1 immunostaining and that both Twist1 and Ets1 are significantly up-regulated in metastatic compared to non-metastatic tumors. Using both mouse and human lung cancer cell lines, we show that Ets1 regulates the expression of Twist1. Chromatin immunoprecipitation assays confirm binding of Ets1 to the Twist1 promoter. Repression of endogenous Ets1 by siRNA demonstrates that mRNA and protein levels of Twist1 are decreased in mouse and human cell lines. siRNA-mediated repression of Ets1 and Twist1 results in decreased migration and invasion in metastatic mouse cell lines.

Several transcription factors, including Twist1, Snail, and SIP1 repress E-cadherin expression and are thought to induce epithelial-mesenchymal transition, a necessary step in tumor metastasis (Yang and Weinberg, 2008). We now show that Ets1 is likely a direct transcriptional activator of Twist1 in lung cancer cell lines. Therapeutic targeting of these E-cadherin repressors/or their regulators, such as Ets1, could be useful to impair EMT and thus metastatic spreading. Identifying upstream Twist1 activators and downstream targets will be critical for understanding the molecular mechanisms underlying metastasis and in finding new targets in cancer treatment. In addition, identification of downstream pathways that are activated during EMT may reveal new diagnostic markers of early stages of lung cancer and possible novel targets for anti-metastatic therapeutics.

References

Please see Appendix 4 for a complete list of references related to manuscript

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Appendices:

1. USCAP Abstract Presentation (submitted 10/13; presentation in 2014)

Correlation of Twist Expression in Different Histologic Subtypes of Primary Adenocarcinoma in Lung

Background: Tumor recurrence and metastasis have significant impact on the mortality of lung cancer. Metastasis is particularly associated with early demise. Transcription factors that mediate the epithelial-mesenchymal transition (EMT) play a major role in facilitating metastasis by a variety of mechanisms. Twist a basic helix-loop-helix transcription factor is well known to be involved in promoting metastasis. The role of Twist in lung cancer has not been studied well due to the lack of a reproducible classification. This has been addressed by the International Association for the Study of Lung Cancer which has published guidelines for a multidisciplinary classification of lung adenocarcinomas that highlights the heterogeneity of lung adenocarcinomas and establishes a uniform classification. In the new system, lung adenocarcinomas are classified as lepidic, acinar, papillary, micropapillary and solid types; each with prognostic significance. This study attempts to correlate Twist positivity with the various subtypes and account for the prognostic variability encountered in the treatment and outcome of patients.

Design: A total of 30 lung adenocarcinomas were subtyped according to the IASLC proposal and stained with Twist monoclonal antibody. Twist staining less than 10% was considered negative, greater than 10% considered positive. Correlation was measured between Twist results and the histologic subtypes.

Result: Kruskal-Wallis analysis on Twist positive cases showed a significant difference among five histologic subtypes ($P < 0.0001$). The most common subtype was Acinar followed by Solid. Chi-square analyses revealed a significant difference in Twist expression in cases that comprise greater than 20% of either or both of these two subtypes.

Conclusion: Twist expression is differentially expressed in the five recognized subtypes of lung adenocarcinoma and may contribute to the biological variability and difference in prognosis observed for these subtypes.

Table1: TWIST expression in subtypes

Histologic Subtypes	Twist (+)	Twist (-)
Lepidic	1	1
Acinar	13	9
Solid	10	7
Papillary	6	2
Micropap	5	1

Table2: TWIST expression in Acinar and Solid

Acinar and Solid	TWIST (+)	TWIST (-)	p-value
>60%	9	6	0.03
>40%	16	7	0.03
>20%	17	9	0.03

2. USCAP Abstract Presentation (2012)

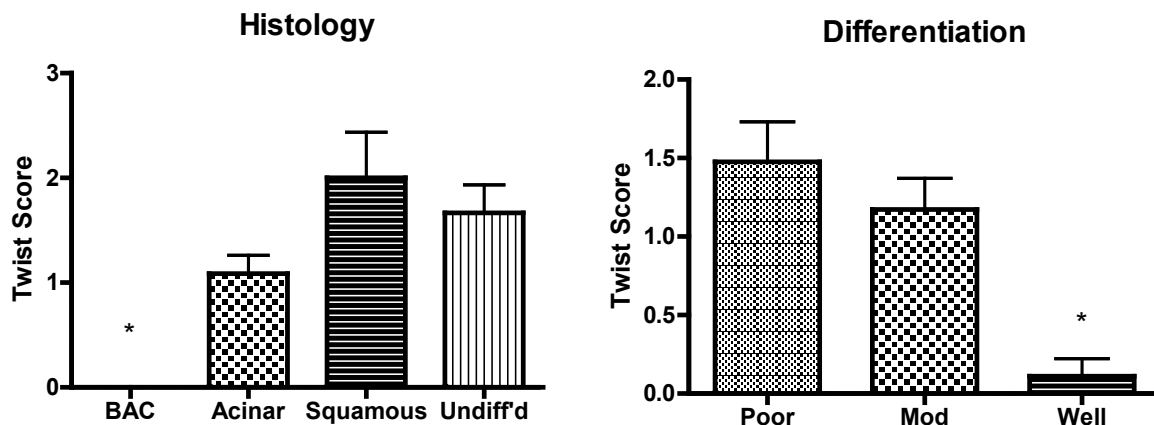
Correlation of Twist Expression in Primary Non Small Cell Lung Carcinomas with Histologic Subtypes, Risk of Metastasis, and Overall Patient Survival

Katherine J. Downey, MD; Yanelba Toribio, MD; Hasmeena Kathuria, MD; Benedict Daly, MD; and Carl O'Hara, MD

Funded by Department of Defense USAMRMC Congressional Directed Medical Research Program (CDMRP) Lung Cancer Program Award (Hasmeena Kathuria)

Tumor metastasis is the primary cause of treatment failure and death for most cancer patients. Transcription factors including, but not limited to, Snail, Zeb1, Slug, and TWIST have been shown to induce epithelial-mesenchymal transition (EMT), a process that is regarded as essential for metastasis. These transcription factors exert their effects mainly through the repression of E-cadherin. TWIST, a highly conserved basic helix-loop-helix (bHLH) transcription factor, is a master regulator of embryonic morphogenesis. Recently, it has been shown to be a key player in mediating cancer metastasis in a number of cancer types such as ovarian cancer, hepatocellular carcinoma, melanoma, prostatic adenocarcinoma, and invasive lobular breast carcinoma. The aim of our study was to evaluate the prognostic value of TWIST expression in patients with resectable non-small cell lung cancers (NSCLC) in relation to staging, risk of metastasis, and different histologic subtypes.

In agreement with recent studies stating prognoses varies with histologic subtypes, our data shows significant difference in Twist expression amongst corresponding subtypes. A significant difference in Twist expression was noted in poorly differentiated adenocarcinomas, as compared to moderately and well-differentiated forms. Twist was found to be over-expressed in tumors with nodal involvement compared to those without. In conclusion, our findings suggest that high Twist expression is an independent marker in NSCLC prognosis, and can be useful in risk stratification and tailoring therapeutic modalities in resectable NSCLC.



* = $p < .05$ well compared to other 3 groups

* = $p < .05$ well compared to Mod or Poor

3. American Thoracic Society Abstract (2011)

ETS-1 Regulates Twist-1 Expression In Non-Small Cell Lung Cancer (NSCLC) Progression And Metastasis

K.K. Wong, MD, PhD, J. Carretero, PhD, M.I. Ramirez PhD, J. Li, A. Hinds, M.C. Williams, PhD, and H Kathuria, MD.

Funded by Department of Defense USAMRMC Congressional Directed Medical Research Program (CDMRP) Lung Cancer Program Award (Hasmeena Kathuria)

Rationale: Metastasis is a multistep process during which cancer cells disseminate from the site of primary tumors and establish secondary tumors at distant organs. One model of metastasis proposes that a subset of epithelial tumor cells acquires the ability to disseminate by converting to a mesenchymal phenotype, a process called EMT. Ets proteins, a group of important transcription factors, act as positive or negative regulators of the expression of genes involved in cellular proliferation, development, and transformation. Elevated Ets1 expression has been observed in many invasive and metastatic tumors including those of lung, breast, and colon. In lung cancer, we hypothesize that Ets1 contributes to lung tumorigenesis by binding to specific promoters that directly control the transcription of genes involved in initiating EMT.

Methods: We used a mouse lung cancer model with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss (KrasG12D /Lkb1L/L model) and a similar model of lung cancer but one that does not metastasize, driven by conditionally activated Kras alone (KrasG12D model). To identify Ets1 and Pea3 targets involved in EMT, we measured expression of Twist, a known EMT-inducer and an Ets1 predicted target, in KrasG12D /Lkb1L/L and KrasG12D cell lines. Using ChIP assays, we determined whether Ets1 and Pea3 bind Twist1 in cell lines derived from KrasG12D versus Kras G12D /Lkb1L/L tumors. We determined whether silencing Ets1 decreases the expression of Twist mRNA and protein.

Results: Ets-1 and Twist clearly differ in gene expression between Kras G12D primary tumor (low Ets-1 and Twist expression), KrasG12D /Lkb1L/L primary tumors (moderate Ets-1 and Twist expression), and KrasG12D /Lkb1L/L metastatic LN specimens (high Ets-1 and Twist expression). ChIP assays confirm binding of Ets1 to the Twist promoter. Repression of endogenous Ets1 by siRNA demonstrates that mRNA and protein levels of Twist are decreased in mouse lung cancer cell lines.

Conclusions: We show a positive correlation between Twist-1 and Ets-1 expression and binding patterns in mouse NSCLC progression. In future studies, we will determine whether silencing Ets proteins and their targets, such as Twist, modulates both in-vitro and in vivo metastatic potential. Identifying upstream Twist activators and downstream targets could be critical in understanding the molecular mechanisms underlying metastasis and in finding new targets in lung cancer treatment.

4. Manuscript in preparation

Ets1 Regulates Twist1 Expression in Non-Small Cell Lung Cancer (NSCLC) Progression and Metastasis

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ABSTRACT

Ets proteins, a group of important transcription factors, act as positive or negative regulators of the expression of genes involved in cellular proliferation, development, and tumorigenesis. In lung cancer, increased Ets protein expression, including Ets1 and Pea3, is associated with poor prognosis. We hypothesized that Ets1 contributes to lung tumorigenesis by binding to specific promoters that directly control the transcription of genes involved in initiating EMT, such as Twist1. We used a mouse lung cancer model with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss (Kras^{G12D}/Lkb1^{L/L} model) and a similar model of lung cancer but one that does not metastasize, driven by conditionally activated Kras alone (Kras^{G12D} model). In this report, we show that Ets1 and Twist1 clearly differ in gene expression between Kras^{G12D} primary tumor (low Ets1 and Twist1 expression), Kras^{G12D}/Lkb1^{L/L} primary tumors (moderate Ets1 and Twist1 expression), and Kras^{G12D}/Lkb1^{L/L} metastatic LN specimens (high Ets1 and Twist1 expression). Similarly, in human lung tumors, Twist1 staining positively correlates with Ets1 immunostaining. Using both mouse and human lung cancer cell lines, we show that Ets1 regulates the expression of Twist1. Chromatin immunoprecipitation assays confirm binding of Ets1 to the Twist1 promoter. Repression of endogenous Ets1 by siRNA demonstrates that mRNA and protein levels of Twist1 are decreased in mouse and human cell lines. siRNA-mediated repression of Ets1 and Twist1 results in decreased migration and invasion in metastatic mouse cell lines. Therapeutic targeting of EMT regulators could be useful to impair tumor metastasis.

Keywords: Lung cancer metastasis, Ets proteins, EMT, Twist1, Ets1

INTRODUCTION

Metastasis is a multistep process during which cancer cells disseminate from the site of primary tumors and establish secondary tumors in distant organs, although other mechanisms (tumor stem cells) of establishing metastases have been proposed. Ets proteins are extremely important in human cancer progression (Seth and Watson, 2005). 27 human Ets family members have been identified, and in some systems multiple Ets factors act in concert to regulate pathways involved in tumorigenesis (Turner et al., 2007).

In lung cancer, increased Ets1 and Pea3 expression, often called 'pro-metastatic' Ets proteins, is associated with poor prognosis (Yamaguchi et al., 2007). Introducing the Pea3 gene into non-small-cell lung cancer (NSCLC) cell lines lacking endogenous Pea3 expression causes increased motility and invasiveness (Hakuma et al., 2005). Elevated Ets1 expression has been observed in invasive and metastatic tumors including those of breast, lung, and colon (Seth and Watson, 2005; Sasaki et al., 2001). The complete pool of Ets1 targets is unknown but Ets1 regulates expression of proteases including urokinase plasminogen activator (uPA) and matrix metalloprotease family members

(Mmps 1, 3, 7, 9 and 13) that are required for tumor cells to degrade and invade surrounding extracellular matrix (Sementchenko and Watson, 2000).

One model of metastasis proposes that a subset of tumor cells within epithelial malignancies acquires the ability to disseminate by undergoing an epithelial-mesenchymal transition (EMT). These cells are characterized by (1) loss of cell-cell attachments, E-cadherin expression, and apical-basal polarity, and (2) appearance of mesenchymal differentiation properties (Huber et al., 2005). EMT is regulated by several transcription factor families, including basic helix-loop-helix factors (Twist1 and E12/E47), zinc finger proteins (Snail1 and Snail2), and two-handed zinc family factors [Sip1 (Zeb2) and Sip2 (Zeb1)] (Kang and Massague, 2005).

To understand the biological processes driving metastasis, genetic mouse models of lung cancer with differing propensities of tumor cells to metastasize have been developed. One model is the $Kras^{G12D}/Lkb1^{L/L}$ lung cancer model driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss that aggressively metastasizes to regional lymph nodes (Ji et al., 2007; Carretero et al., 2010). $Kras^{G12D}/Lkb1^{L/L}$ mice develop tumors within 4 weeks of activation of conditional mutations and die within 8 to 10 weeks, with local and distant metastases. 34% of human lung adenocarcinomas harbor Lkb1 genomic alterations (Sanchez-Cespedes et al., 2002). One function of Lkb1 is to phosphorylate Pea3, targeting it for degradation by a proteasome (Upadhyay et al., 2006). Tissues with Lkb1 mutations have higher Pea3 expression than wild type tissues (Upadhyay et al., 2006). In vitro, Pea3 stabilization through Lkb1 inactivation leads to EMT.

A similar lung cancer model, but one that does not metastasize, is driven by conditionally activated Kras alone ($Kras^{G12D}$ model). Somatic mutations that activate Kras are detected in 30% of human lung adenocarcinomas (Keohavong et al., 1996). $Kras^{G12D}$ mice develop a mixture of adenomas and primary adenocarcinomas, but no metastases, 16 weeks after adeno-Cre activation of oncogenic Kras and die within 24 to 26 weeks (Ji et al., 2007). Previously, mRNA expression profiling of $Kras^{G12D}/Lkb1^{L/L}$ versus $Kras^{G12D}$ primary tumors identified a signature of differentially expressed genes (Ji et al., 2007; Carretero et al., 2010). We mined the available database and confirm increased Ets1, but not Pea3, mRNA expression by QRT-PCR in $Kras^{G12D}/Lkb1^{L/L}$ compared to $Kras^{G12D}$ primary tumors.

We hypothesize that Ets1 contributes to lung tumorigenesis by binding to specific promoters that control transcription of genes involved in EMT. Ets proteins positively or negatively regulate expression of genes involved in proliferation, development, and transformation (Sharrocks, 2001). The regulatory specificity of individual Ets members comes from their cell/tissue patterns of expression, post-translational modifications, interactions with partner proteins, and base-pair differences surrounding the core consensus sequence (Hollenhorst et al., 2005). Ets proteins bind to DNA sequences containing a GGAA/T core motif, with flanking sequences contributing to specificity of different members (Sharrocks, 2001).

We used GSEA and Transfac databases to determine if gene expression differences between $Kras^{G12D}/Lkb1^{L/L}$ versus $Kras^{G12D}$ models were enriched in putative Ets1 targets. We found that Twist1, an Ets1 predicted target, is expressed at higher

levels in $Kras^{G12D}/Lkb1^{L/L}$ compared to $Kras^{G12D}$ primary tumors. In the proximal 2 kb human Twist1 promoter, there are 11 putative Ets sites and in the murine Twist1 promoter there are 34 Ets sites (Qin et al., 2009; Howe et al., 2003). In vitro studies by other investigators show Twist1 over-expression in mammary or prostate cancer cells induces EMT resulting in increased cell migration and invasion (Yang et al., 2006).

In this report, we correlate expression patterns of Twist1 and Ets1 in human and mouse lung cancer specimens by immunohistochemistry. Using mouse and human lung cancer cell lines, we show that Ets1 regulates Twist1 expression. Chromatin immunoprecipitation assays confirm binding of Ets1 to the Twist1 promoter. Silencing Ets1 expression decreases Twist1 mRNA and protein expression. siRNA-mediated repression of Ets1 and Twist1 results in decreased migration and invasion in metastatic mouse cell lines. We speculate that therapeutic targeting of EMT activators and/or their regulators could be useful to impair EMT, and thus tumor metastasis.

RESULTS

Murine $Kras^{G12D}/Lkb1^{L/L}$ primary tumors and metastases, compared to $Kras^{G12D}$ primary tumors, have increased Ets1 mRNA by microarray analysis and both increased Ets1 and Pea3 protein expression by immunohistochemistry

Gene expression microarray analysis show increased Ets1 expression in $Kras^{G12D}/Lkb1^{L/L}$ primary tumors and metastases compared to $Kras^{G12D}$ primary tumors (Fig 1a). To determine if cell types expressing Ets1 differ in the two models, we performed immunohistochemistry on $Kras^{G12D}$ and $Kras^{G12D}/Lkb1^{L/L}$ specimens (Fig 1b). In $Kras^{G12D}$ primary tumors, Ets1 protein expression could not be detected. In $Kras^{G12D}/Lkb1^{L/L}$ primary tumors and metastatic lymph nodes, scattered epithelial cancer cells express Ets1.

Although Pea3 is expressed in both models, there were no differences in Pea3 mRNA expression between $Kras^{G12D}$ and $Kras^{G12D}/Lkb1^{L/L}$ specimens (Fig 1a). Since the normal function of Lkb1 gene is to phosphorylate Pea3, targeting it for proteasome-mediated degradation, we hypothesized that $Kras^{G12D}/Lkb1^{L/L}$ mice might have increased Pea3 protein expression. In $Kras^{G12D}$ primary tumors, Pea3 protein expression could not be detected by immunohistochemistry (Fig 1b). One plausible explanation is that in $Kras^{G12D}$ mice, wildtype Lkb1 phosphorylates Pea3 and either (1) the Pea3 antibody cannot detect $Pea3^{P04}$ or (2) $Pea3^{P04}$ is degraded and therefore not detected. In $Kras^{G12D}/Lkb1^{L/L}$ primary tumors and lymph nodes, scattered epithelial cancer cells express Pea3 (Fig 1b).

Twist1 staining positively correlates with Ets1 and Pea3 immunostaining in mouse lung tumor specimens.

Previously, gene expression microarray analysis showed increased Twist1 expression in $Kras^{G12D}/Lkb1^{L/L}$ compared to $Kras^{G12D}$ tumor specimens (Carretero et al., 2010). We performed immunohistochemistry for Twist1 on $Kras^{G12D}$ and $Kras^{G12D}/Lkb1^{L/L}$ specimens (Fig 1b). Twist1 is not detected in $Kras^{G12D}$ primary tumors. In $Kras^{G12D}/Lkb1^{L/L}$ primary tumors, Twist1 is detected in scattered tumor epithelial cells

(Fig 1b). In metastatic lymph nodes, positive Twist1 staining is detected in tumor epithelial cells and in scattered surrounding stromal cells.

Ets1 binds to the mouse Twist1 promoter.

Cell lines derived from Kras^{G12D} /Lkb1^{L/L} (KW-821) and Kras^{G12D} (KW-634) tumors markedly differ in Twist1 mRNA levels (Fig 2a). KW-821 cells express higher levels of Twist1 mRNA compared to KW-634 cells by QRT-PCR. We performed ChIP assays in KW-821T and KW-821LN cell lines (Fig 2b). Immunoprecipitated samples using IgG or Ets1 antibodies show that Ets1 binds to the Twist1 promoter in both KW-821T and KW-821LN cells. For negative control (Twist1 promoter sequences to which Ets1 does not bind), there is no difference between IgG and Ets1 (data not shown).

Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels.

siRNA mixture targeting Ets1 or non-targeting siRNA control were transfected into KW-821T and KW-821LN cells. We confirm effective knockdown of Ets1 by QRT-PCR and western analysis (Fig 3). Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels in both cell lines (Fig 3).

Silencing either Twist1 or Ets1 decreases migration and invasion in cell lines derived from Kras^{G12D} /Lkb1^{L/L} primary and metastatic cells.

Cells were transfected with siRNAs (siRNA-Ets1, siRNA-Twist1, and siRNA-control), and then seeded in Matrigel-coated invasion or migration chambers for 24 h. Extent of invasion (A) and migration (B) were expressed as an average number of cells per microscopic field. Silencing Twist1 or Ets1 decreases migration and invasion in KW-821 (T and LN) cells (Fig 4).

Twist1 staining is predominately localized to stromal cells adjacent to tumor epithelial cells in human lung cancer specimens.

Twist1 immunohistochemistry was performed on 30 primary human tumors (5 well-, 15 moderate-, and 10 poorly- differentiated adenocarcinomas). Twist1 is detected in 23/30 primary tumors. Twist1 is detected just in stromal cells surrounding tumor epithelial cells in 15/23 specimens. 1 primary tumor specimen stains positive only in tumor epithelial cells. In 7/23 Twist1-positive specimens, staining is detected in both epithelial and surrounding stromal cells (Figure 5a and b). In stromal cells, Twist1 is detected in scattered cells which appear to be fibroblasts by morphology. When Twist1 is detected in epithelial tumor cells, it is preferentially observed in marginal regions of cancer foci and is predominately nuclear. In areas of adjacent normal lung, alveolar epithelial and fibroblast cells do not express Twist1 (data not shown).

15 /30 specimens had patient-matched lymph node metastasis. In all 15 specimens with matched lymph node metastasis, Twist1 is detected in primary tumors, metastatic lymph nodes, or both (Fig 5b). 3/15 patients express Twist1 in just lymph nodes; 3/15 patients stain positive in just primary tumors; and 9/15 express Twist1 in both primary tumor and metastatic lymph nodes. 9/12 metastatic lymph nodes stain

positive in stromal cells surrounding tumor epithelial cells; 3 metastatic lymph nodes stain positive only in tumor epithelial cells. In this small study, we could not determine whether Twist1 protein expression correlates with tumor grade.

Our findings are consistent with previous studies on the expression pattern of Twist1 in primary colorectal cancer, in which Twist1 staining is mostly in stromal cells surrounding tumor epithelial cells (Valdes-Mora et al., 2009). In endometrial cancer, Twist1 is detected in both tumor epithelial and stromal cells (Kyo et al., 2006). In lung cancer, using a polyclonal antibody, among 68 lung cancer specimens, 9 tumors showed weak Twist1 expression; 51 lung tumors showed moderate/strong Twist1 staining, mostly in the cytoplasm of tumor cells, whereas 8 corresponding normal lung specimens demonstrated weak staining (Hui et al., 2009). In their study, Twist1 expression positively correlated with differentiation ($p < 0.002$) (Hui et al., 2009). Hui et al do not describe the pattern of staining we observe in surrounding stromal cells.

Twist1 staining positively correlates with Ets1 immunostaining in human primary tumor specimens.

We characterized the expression pattern of Ets1 and Twist1 in 10 primary tumors (5 primary tumors without LN metastasis; 5 primary tumors with LN metastasis). In primary tumors the expression pattern of Twist1 and Ets1 positively correlate. In Twist1-positive stromal cells, Ets1 expression is detected in stromal cells. In Twist1-positive tumor epithelial nuclei, Ets1 is detected in tumor epithelial nuclei (Fig 5a).

Ets1 binds to the human Twist1 promoter.

We performed ChIP assays in human (A549 and NCI-H1299) cell lines to confirm Ets protein binding to the Twist1 promoter (Fig 6). Sonicated cell extracts were immunoprecipitated using IgG or Ets1 antibodies and analyzed by PCR for the Twist1 promoter spanning the Ets1 site. Figure 6 is a representative gel showing that Ets1 binds to the Twist1 promoter in both cell lines. For negative control (Twist1 promoter sequences to which Ets1 do not bind), there is no difference between IgG and specific antibodies (data not shown).

There are over 30 Ets cis-elements in the mouse Twist1 promoter and 11 Ets cis-elements in the human promoter. Due to variable sizes of promoter DNA fragments generated when DNA is sheared (500 bp to 1kb) in ChIP assays, these experiments can only determine that Ets1 binds the promoter somewhere within the region of average size fragments obtained and cannot identify specific sites that might be utilized preferentially nor can they indicate how many sites are occupied simultaneously.

Silencing Ets1 decreases Twist1 mRNA and protein levels in human cell lines.

We transfected an siRNA mixture targeting Ets1 or non-targeting siRNA control into NCI-H1299 (high Ets1-expressing) cells. We confirm effective knockdown of Ets1 by QRT-PCR and western analysis (Fig 7). Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein levels. Taken together, ChIP and siRNA data confirm that Ets1 is a transcriptional activator of Twist1 in lung cancer cell lines.

Discussion

Little is known about the transcription factors that regulate Twist1 expression in lung and other cancers. To our knowledge, we show for the first time, that in lung cancer cell lines, Ets1 regulates Twist1 expression. We hypothesized that Ets proteins regulate genes involved in EMT such as Twist1, thereby promoting lung cancer progression and metastasis. In the proximal -2 kb of the murine Twist1 promoter, there are 34 putative Ets binding sites, of which approximately one-third is conserved between mouse and human (Qin et al., 2009; Howe et al., 2003). We selected Ets1 as a candidate activator of Twist1 transcription in lung tumor specimens since this transcription factor has been previously implicated in tumor invasion and poor prognosis in lung cancer.

Lymph nodes are often the first site of metastasis in lung cancer. Detection of lymph node metastasis is a key factor in staging and prognosis. For our studies, we used tissue specimens and cell lines derived from a mouse model of lung metastasis driven by conditionally activated Kras with concurrent tumor suppressor Lkb1 loss (Ji et al., 2007). Inactivating mutations of Lkb1 and activating mutations of Kras occur together frequently in human disease (Makowski and Hayes, 2008). Similar to the pattern of LN metastasis in human lung cancer, this model has a 60% penetrance of regional LN and distant metastasis.

Twist1 appears to be a pro-metastasis gene that specifically promotes tumorigenesis by inducing tumor cell EMT. Twist1 is a highly conserved transcription factor belonging to the family of basic helix-loop-helix (bHLH) proteins (Bialek et al., 2004; Cheng et al., 2008). Like many oncogenes, Twist1 is likely to exert similar biological activities during tumor metastasis as it does in normal development. In mice, Twist1 was shown to be required for head mesenchyme, somites and limb buds development (Lee et al., 2009). Mice lacking Twist1 die at E10.5 secondary to defects in cell migration confirming its important role in development and differentiation (Baylies and Bate, 1996). Therefore we anticipated that alteration in Twist1 expression would influence cell migration and/or invasion.

Herein, we report that Ets proteins participate in transcriptional regulation of Twist1 using both murine and human lung cancer cell lines as experimental models. Using the existing mRNA expression database of metastatic versus non-metastatic mouse tumor specimens, we confirm increased Ets1, but not Pea3, mRNA expression by QRT-PCR in Kras^{G12D}/Lkb1^{L/L} (metastatic mouse model) compared to Kras^{G12D} (non-metastatic mouse model) primary tumors.

We determined by ChIP assays that Ets1 binds to the Twist1 promoter in mouse and human cell lines. Silencing Ets1 results in a statistically significant decrease in Twist1 mRNA and protein indicating that Ets1 is likely a direct transcriptional activator of Twist1 in lung cancer cell lines. It is likely that other Ets proteins, cis-elements, and transcription factors are involved in Twist1 regulation in lung tumorigenesis as suggested by studies of these proteins in other cancer cell lines. In mouse mammary epithelial cells, both Pea3 and Ets1 activate the Twist1 promoter in concert with steroid

receptor coactivator-1 (Src-1) (Howe et al., 2003). Src-1 has been found to coactivate Twist1 transcription through physical interactions with Pea3 at the proximal Twist1 promoter, thereby promoting breast cancer metastasis (Qin et al., 2009). The significance of multiple Ets sites within the Twist1 promoter is unknown. In the T β R-II (type II TGF β receptor) promoter 2 Ets sites are required for a synergistic response to Elf3, Ets1, and Pea3 (Kopp et al., 2004), suggesting the likelihood that multiple Ets sites are occupied and active in Twist1 expression.

We characterized the expression pattern of Ets1 and Twist1 in primary tumors and metastatic lymph nodes. Twist1 staining positively correlates with Ets1 immunostaining in both murine and human tissues. Furthermore, Ets1 and Twist1 protein expression match in terms of expressing cells. In Twist1-positive stromal cells, Ets1 expression is detected in the stroma. In specimens in which Twist1 is detected in tumor epithelial nuclei, Ets1 is detected in tumor epithelial nuclei. In some cases, the tumor epithelial cells are strongly stained, particularly at the margins of the tumor.

We show that Twist1-positive cells are preferentially localized in stroma adjacent to clusters of cancer cells. Although these cells have a fibroblast-like morphology, it is possible that they are, or are derived from cancer cells that have undergone a morphological change via EMT. Modified fibroblasts, variably called myofibroblasts, reactive stroma, or cancer-associated fibroblasts, are thought to play a central role in tumor-stroma interactions (Kalluri and Zeisberg, 2006). The concept that cancer cells become metastatic via EMT is based on observations that acquisition of mesenchymal markers [such as vimentin and S100A4 (also known as fibroblast-specific protein 1 (FSP1))] by epithelial carcinoma cells and loss of epithelial cell adhesion molecules [such as E-cadherin] is associated with increased metastatic potential. Further characterization of these Twist1-positive epithelial and stromal cells will be the focus of future studies.

Previous studies in NCI-H1299 cells show that siRNA-mediated Twist1 repression decreases N-cadherin and increases E-cadherin expression resulting in decreased migration and invasion (Yang et al., 2008). We now show that silencing Twist1 or Ets1 decreases migration and invasion in both Kras^{G12D}/Lkb1^{L/L} primary and metastatic-derived cell lines.

Several transcription factors, including Twist1, Snail, and SIP1 repress E-cadherin expression and are thought to induce EMT (Yang and Weinberg, 2008). Among the transcription factors we examined, Twist1 and Ets1 are significantly up-regulated in metastatic compared to non-metastatic tumors. Therapeutic targeting of these E-cadherin repressors/or their regulators, such as Ets1, could be useful to impair EMT and thus metastatic spreading. Identifying upstream Twist1 activators and downstream targets will be critical for understanding the molecular mechanisms underlying metastasis and in finding new targets in cancer treatment.

MATERIALS AND METHODS

Gene expression Microarray Analysis

Gene expression profiles comparing Kras^{G12D} primary tumors (9 tumors), Kras^{G12D}/Lkb1^{L/L} primary tumors (9 tumors) and Kras^{G12D}/Lkb1^{L/L} metastasis (16 lymph node and distant sites) have been previously characterized (Carretero et al., 2010). As described by Carretero et al, using a two-class unpaired differential expression analysis and a false-discovery rate (FDR) of < 0.01, signatures reflecting (1) gene expression changes induced by Lkb1 loss in primary tumors (Kras^{G12D} versus Kras^{G12D}/Lkb1^{L/L} primary tumors) and (2) changes associated with metastases (Kras^{G12D}/Lkb1^{L/L} primary tumors versus Kras^{G12D}/Lkb1^{L/L} metastases) were generated (Carretero et al., 2010). We used these signatures to compare Ets1 and Pea3 gene expression changes in (1) Kras^{G12D} primary tumors, (2) Kras^{G12D}/Lkb1^{L/L} primary tumors, and (3) Kras^{G12D}/Lkb1^{L/L} metastasis.

Immunohistochemistry

Archived paraformaldehyde-fixed, paraffin-embedded human lung adenocarcinomas were studied. A pathologist reviewed H&E stained sections and assigned a grade of well-, moderate-, or poorly-differentiated tumors (Travis et al., 1999). Thyroid transcription factor (Nkx2.1, TTF-1) immunohistochemistry confirmed lung origin of specimens (Kaufmann et al., 2000). Antigen retrieval for Ets1 and Twist1 is Vector Antigen Unmasking Solution (H3300) (microwave low-power, 15 min) (Ge et al., 2006; Namimatsu et al., 2005). Sections were incubated with primary antibody [mouse monoclonal anti-Ets1 (sc-56674, Santa Cruz Biotechnology) or mouse monoclonal anti-Twist1 (sc-81417, Santa Cruz Biotechnology)] in PBS (4°C, 16 hr). Antibody binding was detected using Vectastain Elite ABC kit with DAB as the substrate. Control slides lacking primary antibody were included in all procedures. Sections were counterstained with methyl green, hematoxylin, or left unstained and photographed in a Leitz Aristopan microscope using ImagePro software. Photographs shown are representative from n=30 samples for Twist1 and n=15 for Ets1.

For mouse lung cancer specimens (n=3 for each model), formalin-fixed paraffin embedded tissue were sectioned at 5 µm. Slides were deparaffinized in xylene and rehydrated sequentially in ethanol. Similar methods were used for TTF-1, Twist1, Ets1 and Pea3 [mouse monoclonal anti-PEA3 antibody (sc-113, Santa Cruz Biotechnology)] immunostaining except that antibody binding was detected using Vector Mouse on Mouse Kit with DAB as the substrate.

Characterization and Culture of the Cell Lines

Human NSCLC cell lines were selected based on mRNA and protein analyses showing that NCI-H1299 cells (metastatic lymph node-derived) express high Ets1 levels (Sloan et al., 2009); A549 cells (primary lung carcinoma-derived) express lower Ets1 levels (Ko et al., 1999)

Tumor-derived cell lines from mice with different abilities to generate lymph node metastases were used. KW-634 cells are primary tumor-derived cell lines from Kras^{G12D}

mice; KW-821 (T) cells are primary tumor-derived and KW-821 (LN) cells are metastatic lymph node-derived cell lines from Kras^{G12D} /Lkb1^{L/L} mice.

NCI-H1299, KW-634 and KW-821 (T and LN) cells were maintained in RPMI 1640 medium (Invitrogen) with 10 mM HEPES; A549 cells were maintained in DMEM (Invitrogen). Cells were maintained in media containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate, incubated at 37°C in 5% CO₂, and harvested at 80% confluence for experiments.

RNA Purification

Total RNA was isolated from cell lines with TRIzol (Invitrogen) and treated for DNA contamination using DNA-free (Ambion). 500 ng RNA was reverse-transcribed using TaqMan reagents (Applied Biosystems) [25°C, 10 min; 37°C, 60 min; 95°C, 5 min].

Real time RT-PCR

Twist1 and Ets1 mRNA expression were analyzed by quantitative real time RT-PCR (QRT RT-PCR) using ABI Prism 7000 sequence detector (Applied Biosystems). Reverse transcriptions were diluted 1:25. Primers and probe sequences are as follows: Twist1 (h): F 5'CTAATCCAAGCATCCCTTTGCC3', R 5'TTTATTACTGCC TCCTCCCCCA3'; β-actin: F 5'CCCTGAAGTACCCCATCGAG3', R 5'CAGATTTTCTCCATGTCTG TCCC3', Probe 5'ACGGCATCGTCACCA3'. For Ets1 (h), TaqMan gene expression assay Hs00901425_m1 (Applied Biosystems) was used. For mouse studies, TaqMan assays are as follows: (Twist1: Mm00442036_m1; Ets1: Mm00468970_m1). Reactions were performed in 50 µl and amplified (95°C, 10 min; 40 cycles: 95°C x 15 sec and 60°C x 1 min) using SYBR Green or TaqMan PCR Master Mix (Applied Biosystems). Relative gene expression levels were normalized to β-actin. We quantified relative gene amplification using comparative threshold cycle method ($2^{-\Delta\Delta CT}$). Experiments were performed in duplicate. Data were analyzed by student's t-test with $p < 0.05$ significant.

Protein Purification, Western Blots, and Protein Densitometry

Cell monolayers were trypsinized, washed in PBS, centrifuged, resuspended in lysis buffer with protease inhibitors, and incubated with rotation (4°C, 60 min). Lysate was centrifuged (10 minutes, 13,000 rpm, 4°C). Supernatant (20-50 µg protein) was electrophoresed on a 12% polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked in 1X TBST containing 5% dry milk (1h, RT), exposed overnight (4°C) to mouse anti-Twist1 antibody (1:1000) followed by anti-mouse secondary antibody (1:10000, 1h, RT) or rabbit polyclonal anti-Ets1 antibody (1:1000) (sc-350, Santa Cruz Biotechnology) and anti-rabbit secondary antibody (1:20000). Immunoblots were probed for β-actin to control for equal loading. Binding of labeled horseradish peroxidase-secondary antibodies was detected with Super-Signal West Pico Chemiluminescent Substrate (Pierce). All experiments were performed in triplicate.

Chromatin Immunoprecipitation Assays

Cells were fixed with 1% formaldehyde, incubated (37°C, 15 mins), washed with PBS, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8, 1 mM

PMSF, 1 mM pepstatin A, and 1 mM aprotinin), and sonicated on ice to 500 to 1000 base-pair fragments (Power 5, 5 cycles of 5 mins, 25 s on, 5 s off; Fisher Scientific sonicator). Lysate was centrifuged (RT, 4000 rpm, 5 mins). Supernatant was divided into aliquots, one aliquot stored as input DNA. 3 µg of anti-Ets1 or nonspecific IgG (mouse IgG for Ets1) antibodies were added to other aliquots. Dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, and 150 mM NaCl, 1 mM PMSF, 1 mM Aprotinin) was added and samples were incubated (4°C, overnight) with rotation.

AG beads (Santa Cruz Biotechnology, Inc.), BSA (100 µg/ml), and salmon sperm (500 µg/ml) were added to samples. Samples were rotated (4°C, 2 hrs) and centrifuged (4000 rpm, 2 mins). Pellets were washed in buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 1 mM PMSF, 1 mM Aprotinin), then with final wash buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8). Immune complexes were eluted with buffer (1% SDS, 100 mM NaHCO₃), incubated with rotation (RT, 15 mins), and centrifuged (4000 rpm, 2 mins). Proteinase K (500 µg/ml) and RNase A (500 µg/ml) were added to supernatants and input DNA, then incubated (37°C, 30 mins). Cross-links were reversed (65°C, overnight) and DNA was purified.

DNA fragments were analyzed by PCR for Twist1. Input DNA served as positive controls; IgG-precipitated DNA and human/mouse Twist1 promoter sequences to which Ets1 do not bind served as negative controls. Primers for mouse Twist1 ETS site: 5'GGTTCGCAGGACGAATTGTT3'; 5'AGGGACTTTTCGAAGTTTATAGGAA3'. Primers for human Twist1 ETS site: 5'AGCCCCAGCAATCCAAATC3'; 5'GATGTCTCATCTCGCCCAAGAG3'. Negative control primers: (human: 5'CTGCAGACTTGGAGGCTCTT3'; 5'GTCAGGCCAATGACACTGCT3'; mouse: 5'TGGACAGAGATTCCCAGAGG3'; 5'CTATCAGAATGCAGAGGTGTGG3').

Ets1 and Twist1 small interfering RNA transfection (siRNA)

KW-821 (T4 and LN) cell lines derived from Kras^{G12D}/Lkb1^{L/L} mice were used. 2-3 x 10⁵ cells/35-mm dish were transfected with 1 µmol/L Accell SMARTpool siRNA-Ets1 or siRNA-Twist1 (Thermo Fisher Scientific) mixed with Accell siRNA Delivery Media (Thermo Fisher Scientific). Accell Non-targeting Pool (siRNA-ctr) and Accell delivery media alone served as controls for non-sequence-specific effects.

For human studies, ON-TARGETplus SMARTpool siRNA mixtures targeting Ets1 (L-003887-00) and non-targeting control (D-001810-10) were obtained (Dharmacon). 2-3 x 10⁵/35-mm dish NCI-H1299 (high-Ets1 expression) cells were treated with 50 nM siRNA mixture targeting Ets1 or non-targeting control using DharmaFECT transfection protocol.

siRNA-treated cells were cultured (standard conditions) for 48-72 hrs. RNA was isolated, DNase treated, reverse transcribed, and analyzed by QRT RT-PCR. Protein was isolated, electrophoresed, and analyzed by Western blot. Data (n=3) are expressed as average fold difference between targeting siRNA compared to non-targeting control siRNA (control siRNA designated as 1).

Invasion and Migration Assay

The effects of Twist1 and Ets1 on migration and invasiveness in KW-821 (T4 and LN) cell lines were tested using quantitative CytoSelect™ 24-well Cell Migration and Invasion Assay (Cell Biolabs). Cells were seeded 24 hours after transfection with siRNAs (siRNA-Ets1, siRNA-Twist1, siRNA-control) and migration and invasion assays were performed for another 24 hours.

Invasion assays were performed in an invasion chamber consisting of a cell culture insert (8 µm pore size coated with reconstituted basement membrane matrix) placed in a tissue culture well. Mouse cells suspended in SFM (density of 5×10^5 cells/ml) were seeded into culture inserts 24 hours after siRNA transfection. Cells were allowed to invade basement membrane matrix for 24 h. Invaded cells were stained with CyQuant® GR Dye (Invitrogen) and counted.

For migration assays, cell suspension containing 5×10^5 cells/ml in SFM was placed in polycarbonate membrane inserts (8 µm pore size) 24 hours after siRNA transfection and allowed to migrate through membrane pores. After 24 hours, migratory cells were dissociated from the membrane, detected by CyQuant® GR Dye (Invitrogen) and counted.

Migration and invasion assays were performed in duplicate over two separate experiments for each cell line and siRNA condition. Three random fields were chosen for each membrane, and migration and invasion extent were expressed as average number of cells/microscopic field.

Conflict of Interest

The authors declare no conflict of interest.

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Fig. 1a

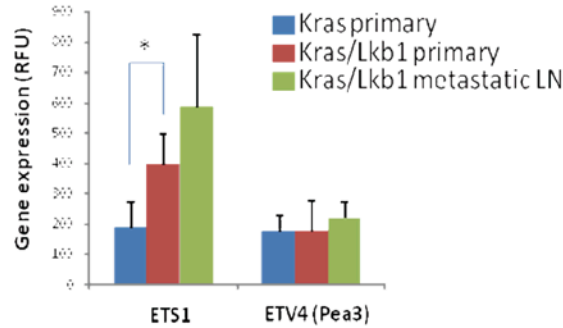


Figure 1a. Microarray data comparing *Ets1* and *Pea3* in $Kras^{G12D}$ primary tumors (blue bar), $Kras^{G12D}/Lkb1^{L/L}$ primary tumors (red bar), and $Kras^{G12D}/Lkb1^{L/L}$ metastatic lymph nodes (green bar). Signatures reflecting gene expression changes induced by *Lkb1* loss in primary tumors ($Kras^{G12D}$ primary tumors versus $Kras^{G12D}/Lkb1^{L/L}$ primary tumors) and changes associated with metastases ($Kras^{G12D}/Lkb1^{L/L}$ primary tumors versus $Kras^{G12D}/Lkb1^{L/L}$ metastases) have been previously generated using two-class unpaired differential expression analysis and an FDR of < 0.01 . Using these signatures, *Ets1* and *Pea3* gene expression was compared in $Kras^{G12D}$ and $Kras^{G12D}/Lkb1^{L/L}$ specimens. *Ets1* gene expression clearly differs in expression between $Kras^{G12D}$ primary tumor (low *Ets1* expression), $Kras^{G12D}/Lkb1^{L/L}$ primary tumors (intermediate *Ets1* expression), and $Kras^{G12D}/Lkb1^{L/L}$ metastatic LN specimens (high *Ets1* expression). *Pea3*, on the other hand, does not appear to differ in expression between primary tumors and metastatic lymph nodes. (*) indicates $p < 0.05$.

Fig. 1b Mouse Primary Tumor and Lymph Node Specimens

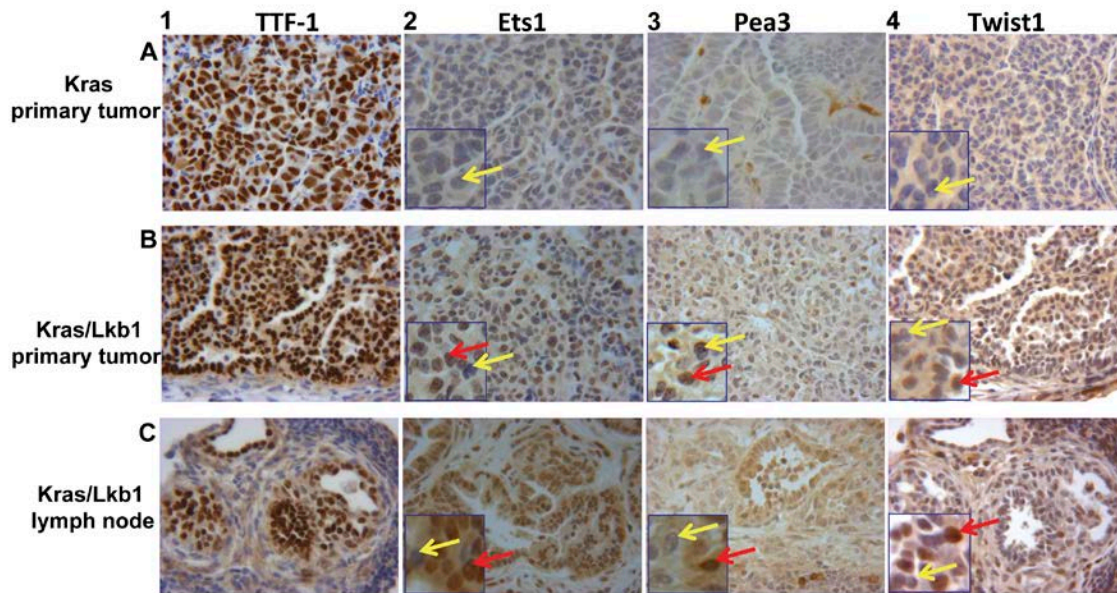


Figure 1b. Immunohistochemical localization of Ets1, Pea3, and Twist1 in mouse lung tumor specimens. (A) Kras primary lung tumor (40x; 100x). (1) TTF-1 staining is localized to tumor cells. (2) Neither tumor foci nor surrounding stromal cells express detectable Ets1. (3) Tumor epithelial cells do not express detectable levels of Pea3. (4) Similar to Ets1 and Pea3, tumor cells express little or no Twist1. (B) Kras/Lkb1 primary lung tumor (40x; 100x) (1) TTF-1 staining. (2, 3) In Kras^{G12D}/Lkb1^{L/L} primary tumor specimens, there are more epithelial cancer cells that express Ets-1 and Pea3 compared to Kras tumor cells. (4) Similar to Ets1 and Pea3, scattered tumor epithelial cells express Twist1. (C) Kras/Lkb1 metastatic lymph node (40x; 100x) (1) TTF-1 staining. (2, 3) In metastatic lymph nodes, most tumor cells express Ets-1 and Pea3. (4) Twist staining positive in scattered epithelial cells and in scattered stromal cells surrounding tumor foci. [yellow arrows, negative cells, purple nuclei; red arrows, positive cells, brown nuclei].

Fig. 2

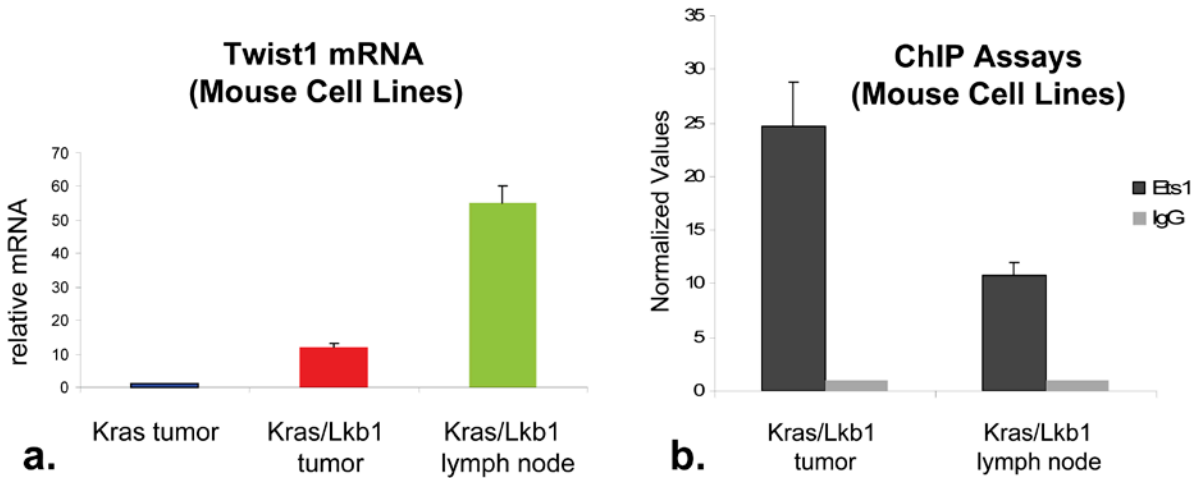


Figure 2. (a) Cell lines derived from $Kras^{G12D}/Lkb1^{L/L}$ model express higher levels of Twist mRNA compared to cell lines derived from $Kras^{G12D}$ model. (A) QRT-PCR analysis shows that Twist mRNA is significantly higher in $Kras^{G12D}/Lkb1^{L/L}$ cells compared with $Kras^{G12D}$ cells. Relative gene amplification calculated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$). Values expressed as fold difference between $Kras^{G12D}/Lkb1^{L/L}$ and $Kras^{G12D}$ cells (with $Kras^{G12D}$ mRNA levels designated as 1). Data are expressed as the mean of 3 assays \pm SE. mRNA levels are normalized to β -actin. **(b)** ChIP analyses in cell lines derived from $Kras^{G12D}/Lkb1^{L/L}$ primary tumor and metastatic lymph nodes show that Ets1 binds to the Twist promoter. Representative ChIP assay using IgG control or antibodies against Ets1. After immunoprecipitation, samples were analyzed by Q-PCR (normalized to input) for Twist promoter fragments spanning the ETS site. Ets1 binds to the Twist promoter in $Kras^{G12D}/Lkb1^{L/L}$ cells derived from primary tumor and metastatic lymph nodes. Data are expressed as the mean of 3 assays \pm SE.

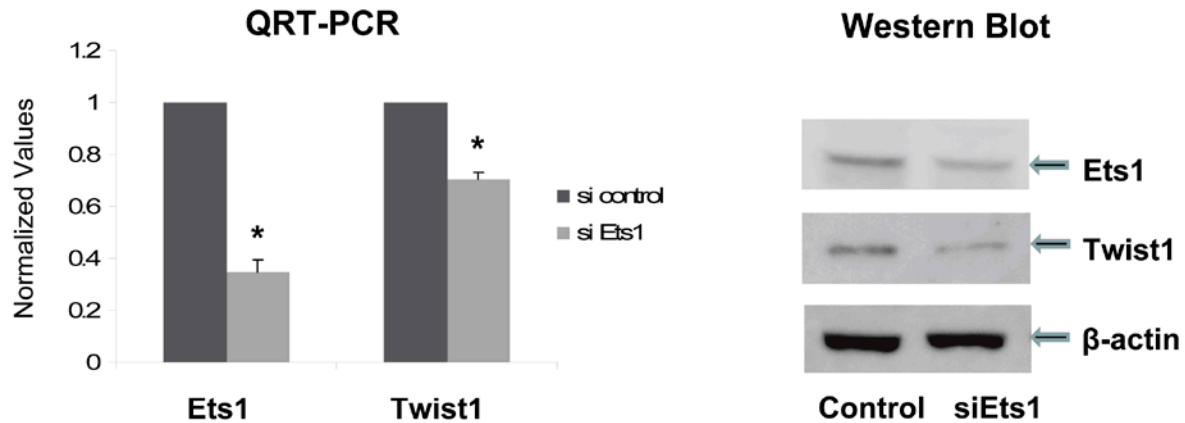
Fig. 3**Kras/Lkb1 Primary Tumor Cell Lines**

Figure 3. Silencing Ets1 results in significantly decreased Twist mRNA and protein expression in cell lines derived from both Kras^{G12D}/Lkb1^{L/L} primary tumor and metastatic lymph nodes. Representative data from Kras^{G12D}/Lkb1^{L/L} primary tumor cell lines are shown. Cells were transfected with siRNA solution targeted to Ets1 (si Ets1) or to non-targeting siRNA control (si control) for 48-72 hrs. Total RNA and protein from transfected cells were purified for analysis by QRT-PCR and Western blot. (A) QRT-PCR shows Ets1 mRNA is effectively decreased in cells transfected with Ets1 siRNA compared to control siRNA. Twist mRNA expression is decreased in cells transfected with Ets1 siRNA compared with control siRNA. (B) Representative Western blot show that silencing Ets1 decreases Twist protein expression. Equal amounts of cell lysates were blotted for Ets1, Twist and β-actin. All data are expressed as the mean of 3 assays +/- SE. (*) indicates $p < 0.05$. Data are expressed as fold difference between Ets1 siRNA compared to control siRNA (with control siRNA designated as 1).

Fig. 4

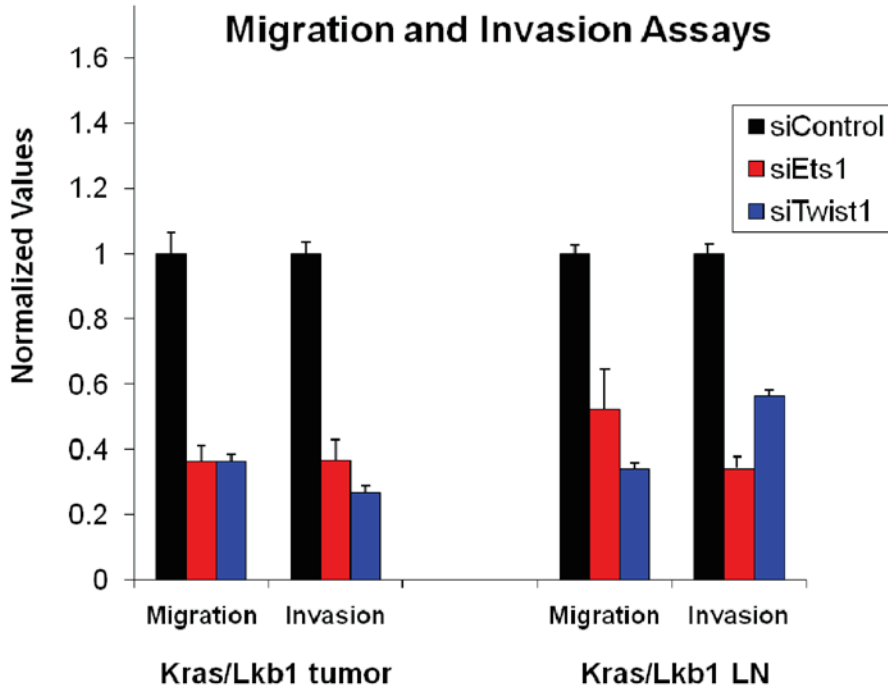


Figure 4. Silencing either Twist1 or Ets1 decreased migration and invasion in cell lines derived from both Kras^{G12D}/Lkb1^{L/L} primary tumor and metastatic lymph nodes. Cells were transfected with siRNAs (siRNA-Ets1, siRNA-Twist siRNA-control), and then seeded in Matrigel-coated invasion chambers or migration chambers for 24 h. Migration and invasion assays were performed in duplicate over a minimum of three separate experiments for each cell line and siRNA condition. The extent of (A) invasion (B) and migration were expressed as an average number of cells per microscopic field. Data is graphed as mean of three replicates and standard deviation (\pm SE) and are representative of two independent experiments.

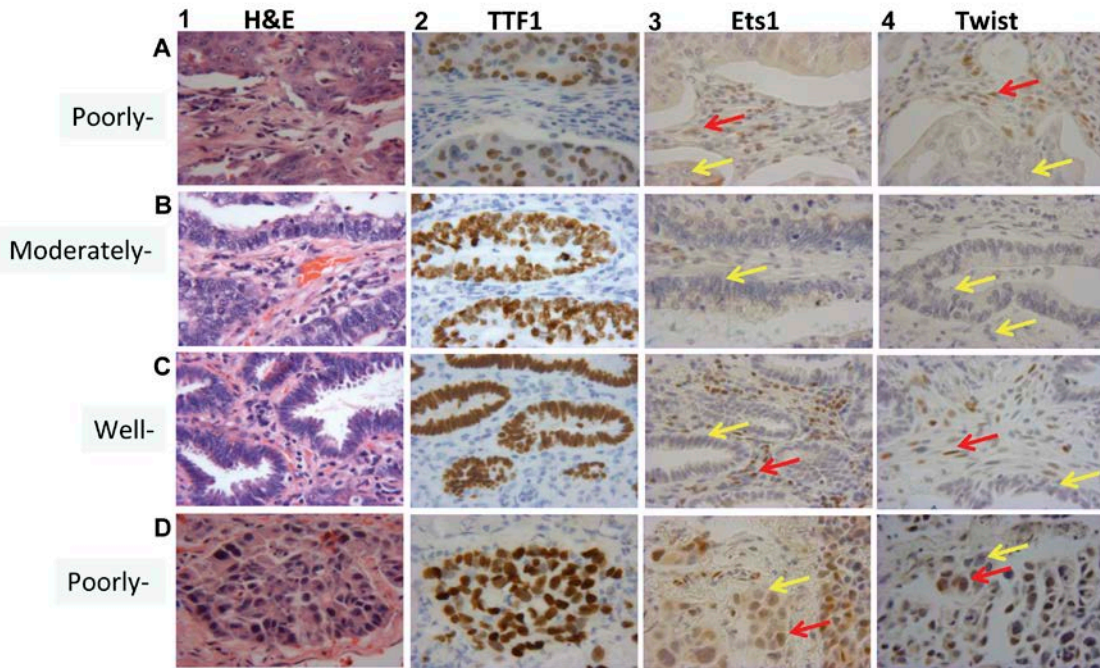
Fig. 5a**Human Primary Lung Tumors**

Figure 5a. Immunohistochemical localization of Twist1 and Ets1 in human lung adenocarcinomas. (A) Poorly-differentiated tumor (1, H&E staining, 40x). (2) TTF-1 staining is localized to tumor cells. (3) Tumor cells express little or no Ets1. Scattered surrounding stromal cells stain positive for Ets1. (4) Similar to Ets1 expression, surrounding stromal cells express Twist1. (B) Moderately-differentiated tumor (1, H&E staining, 40x; 2, TTF-1 staining). (3) Neither tumor foci nor surrounding stromal cells express detectable Ets1. (4) Little or no Twist1 staining in tumor foci or surrounding stromal cells. (C) Well-differentiated tumor (1, H&E staining, 40x; 2, TTF-1 staining). The pattern of expression is similar to panel A, with (3) little or no Ets1 staining in tumor cells, and positive staining in scattered stromal cells (4) Twist1 staining mostly negative in tumor cells, and positive in stromal cells surrounding tumor foci. (D) Poorly-differentiated tumor (1, H&E staining, 40x; 2, TTF-1 staining). Tumor epithelial cells exhibit positive staining for (3) Ets1 and (4) Twist1. [yellow arrows, negative cells, purple nuclei; red arrows, positive cells, brown nuclei]

Fig. 5b

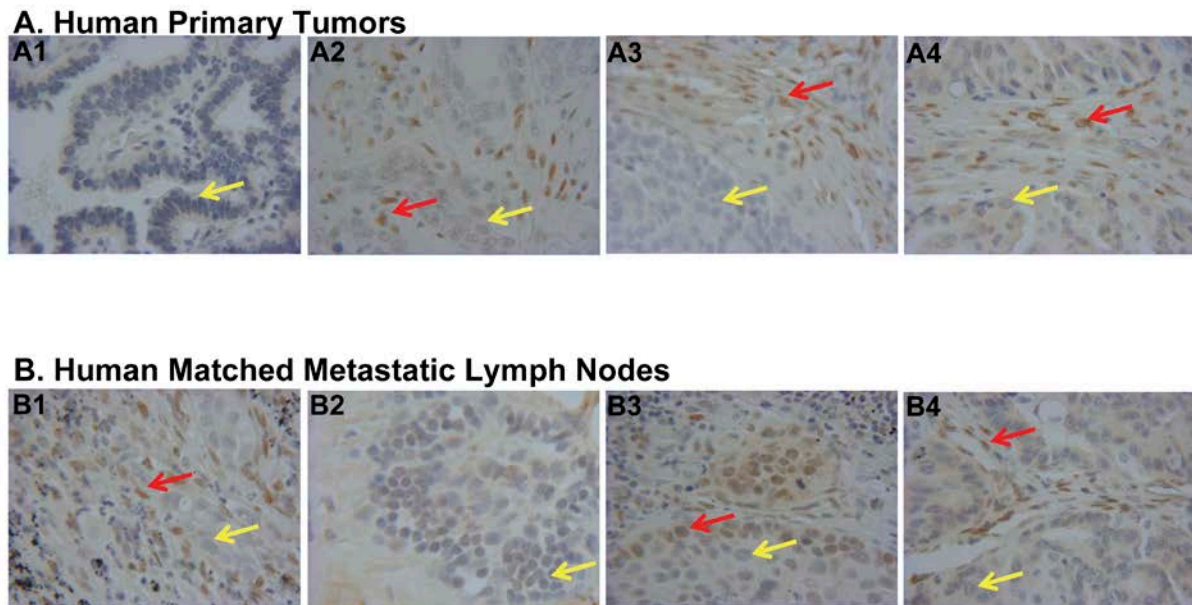


Figure 5b. Representative results for Twist1 immunostaining of paired (A) primary tumors and (B) matched metastatic lymph nodes. (A1) In the primary tumor specimen, Twist is neither expressed in tumor foci nor surrounding stromal cells; whereas (B1) in the corresponding lymph node, Twist is expressed in adjacent stromal cells. (A2) Conversely, immunostaining for Twist is positive in nuclei and stromal cells of primary tumor, but (B2) negative in matched metastatic lymph node. (A3-4; B3-4) High-power view of primary tumor (A3,A4) and metastatic LN (B3,B4) shows positive Twist1 staining in nuclei of tumor cells and/or surrounding stromal cells in both primary tumor and metastatic lymph node. Surrounding lymphocytes are negative. [yellow arrows, negative cells, purple nuclei; red arrows, positive cells, brown nuclei]

Fig. 6

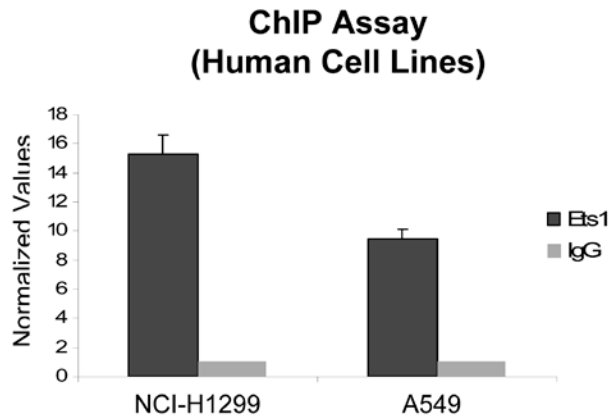


Figure 6. ChIP assays were performed with the indicated antibodies (Ets1 or IgG) and isolated DNA analyzed by qPCR using Twist1 primers. ChIP binding analysis in A549 and NCI-H1299 shows that Ets1 binds to the Twist promoter in both cell lines. Representative ChIP assay of the human Twist1 promoter using IgG control or antibodies against Ets1 in NCI-H1299 or A549 cells. After immuno-precipitation, samples were analyzed by Q-PCR (normalized to input) for Twist promoter fragments spanning the ETS site (n=3). All data are expressed as the mean of 3 assays +/- SE. For negative control (Twist1 promoter sequences to which Ets1 do not bind), there is no difference between IgG and the specific antibodies as a control (data not shown).

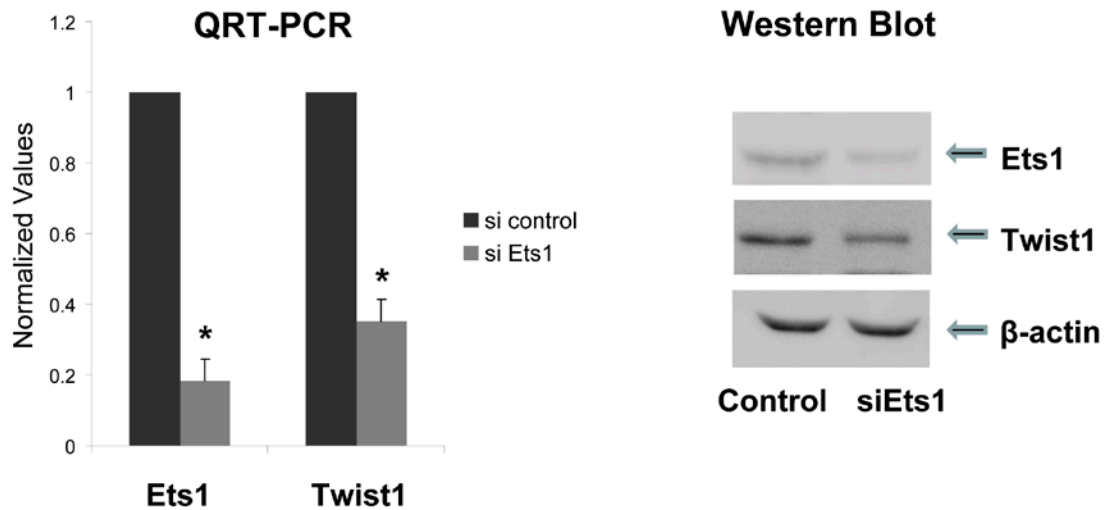
Fig. 7**Human Cell Lines
(1299 Cells)**

Figure 7. Silencing Ets1 results in significantly decreased Twist1 mRNA and protein expression in NCI-H1299 cells. (A,B) NCI-H1299 cells were transfected with siRNA solution targeted to Ets1 (si Ets1) or to non-targeting siRNA control (si control) for 48-72 hrs. Total mRNA and protein from transfected cells were purified for analysis by QRT-PCR and Western blot. (A) QRT-PCR shows Ets1 mRNA is effectively decreased in cells transfected with Ets1 siRNA compared to control siRNA. Twist mRNA expression is decreased in cells transfected with Ets1 siRNA compared with control siRNA. (B) Representative Western blot show that silencing Ets1 decreases Twist1 protein expression. Equal amounts of cell lysates were blotted for Twist1 and β -actin. All data are expressed as the mean of 3 assays \pm SE. Data are expressed as fold difference between Ets1 siRNA compared to control siRNA (with control siRNA designated as 1). (*) indicates $p < 0.05$.